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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The breast produces inhibitors of mammary tumor formation. We hypothesized increases in the amount of these compounds would delay cancer onset. We study the molecule TGF-β, which blocks cell growth. TGF-β is produced as latent complex consisting of the TGF-β homodimer, the TGF-β propeptide dimmer, and a second gene product, the latent TGF-β binding protein (LTBP). LTBP targets latent TGF-β to the extracellular matrix, from which active TGF-β is released. The third cysteine rich repeat (CR3) of LTBP-1 is necessary and sufficient for covalent interaction with small latent TGF-β. CR3 overexpression should result in the TGF-β propetide complexing to an LTBP form unable to interact with matrix. Therefore, TGF-β in this complex should be more easily activated. We generated mice overexpressing CR3 under control of breast specific WAP promoter, and will generate mice overexpressing CR3 under control of MMTV LTR. We will study whether breast cancer occurrence is delayed compared to wt animals. We will test whether tamoxifen treatment, which prevents breast cancer, and overproduction of TGF-β in genetically engineered mice block tumorigenesis better than either condition alone.

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FOREWORD

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Branka Jabonc 7/24/01

PI - Signature Date

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Introduction

TGF-β is the most potent inhibitor of the progression of normal mammary epithelial cells through the cell cycle (Robinson et al. 1991). During the early stages of breast cancer development, the transformed epithelial cells are sensitive to TGF-β-mediated growth arrest, and TGF-β acts as an anti-tumor agent. We hypothesized that, if methods were found that increased the amount of naturally produced TGF-β, the onset of cancer might be delayed or prevented. TGF-β is normally produced in an inactive form and must be freed from the inactive complex to be active. The latent TGF-β complex consists of the TGF-β homodimer plus its N-terminal precursor propeptide dimer and is named small latent complex (SLC) (Derynck et al., 1985; McMahon et al., 1996; Miller et al., 1992). The precursor propeptides are disulfide bonded to a second gene product, the latent TGF-β binding protein (LTBP) (Kanazaki et al., 1993), and this complex is called the large latent complex (LLC). Most cells (such as T-47-D breast cell carcinoma cells) produce TGF-β as part of the LLC (Miyazono et al., 1993; Sporn et al., 1992; Massagué et al., 1992; Harpel, 1998). LTBP targets latent TGF-β to the extracellular matrix, from which biologically active TGF-β may be released. Our lab demonstrated that the third cysteine rich repeat (CR3) of LTBP-1 is necessary and sufficient for covalent interaction with small latent TGF-β (Gleizes et al., 1996). We reasoned that overexpression of this domain, CR3, should result in all of the TGF-β propeptide complexing to an LTBP-1 form that is unable to interact with matrix and therefore would be more easily activated. Using HT1080 human fibrosarcoma cells as a model for the secretion and proteolytic release of pericellular matrix-associated TGF-β (Taipale et al., 1992; Taipale et al., 1994), we have shown that the ECR3E construct, which consists only of the domain that is bound to the TGF-β propeptide plus flanking EGF-like repeats, binds to TGF-β, and that TGF-β in this complex is not incorporated into the

matrix (Mazzieri, unpublished results). We have also shown that overexpressing the ECR3E results in excess latent TGF-β activation in the skin, and we proposed to characterize its activities when overexpressed in the breast. I proposed to generate genetically engineered mice that expressed CR3 under the control of breast-specific MMTV and WAP promoters. This should increase the local concentration of TGFβ in mammary tissue and suppress mammary tumor formation. As the anti-cancer agent tamoxifen is believed to enhance latent TGF-β activation, we also proposed to determine if tamoxifen acted synergistically in delaying tumors in transgene vs. normal mice.

For the second year of this project, we proposed to breed the CR3 transgenes into a strain of mice that is susceptible to tumor induction and to test whether transgene expression affects tumor induction. Although we have experienced some delays as well as some unforeseen experimental difficulties, we have made substantial progress towards our proposed goals.

Construction of the MMTV-ECR3E Transgene.

As described in the previous report, our initial attempts to generate this transgene were unsuccessful. As proposed last year, we obtained a new MMTV promotor construct from Dr. Pamela Cowin, NYU School of Medicine. This promotor has the advantage that it should allow directional cloning using a unique SalI-Hind III MMTV fragment. We performed several ligations and assayed the resultant plasmids, but found that the MMTV promotor was absent from all bacterial colonies. We currently think that for some unknown reason the bacteria used for transformation do not maintain clones that contain both the MMTV and the ECR3E

fragments oriented in the proper direction. It is possible that this combination is toxic to the bacteria or some sequence combination triggers the bacteria to excise the MMTV fragment.

We will continue in the next year to attempt to construct this transgene. We will search for another MMTV promotor construct that may permit construction of the transgene.

Establishment of MMTV-neu/WAP-ECR3E Mice.

Also as described last year, we were not able to obtain the MMTV-pYMT #121 mice originally described in the application as these animals were no longer being bred by the investigators who developed them. Instead, we decided to use MMTV-neu #202 mice that are commercially available and develop breast tumors at 140 days with 100% penetrance. We obtained these mice at the beginning of this reporting period. The animals had to be rederived to move them into our SPF facility and a colony had to be established. This has been done. Crosses between the MMTV-neu and WAP-ECR3E animals have been made, and we are currently genotyping and backcrossing to establish breeding pairs with the correct genotypes. We will utilize these animals to analyze tumor induction in the next year. The initial studies will include whole mount staining and histological analysis. Later studies will include survival curves and analysis of tumor development under baseline and tamoxefin-treated conditions.

Effects of ECR3E on Mammary Gland Development.

As stated in the previous report, we were able to produce the WAP-ECR3E transgene. We used this transgene to produce transgenic animals. Initially, five WAP transgenic lines were established, three of which were chosen for additional studies. The WAP gene is normally expressed during lactation and transiently during the estrus cycle. To determine if the transgene

had an effect on duct formation in non-lactating animals, the morphology of the mammary tissue was examined during the estrus cycle in virgin mice of different age. Mice determined to be in estrus were sacrificed by affixation and the mammary glands removed, spread over a microscopic slide and fixed overnight in Carnoy's fixative. The next day, the specimens were hydrated and stained overnight in Carmin alum stain, which stains the epithelial ducts. After staining, the samples were dehydrated in ethanol, cleared in xylene and mounted with Paramount.

The mammary glands from 4-week old virgin wild type and transgenic animals did not show any significant difference in the appearance of the ductal branching or alveoli formation. At this time, the ductal system has just started to form and occupies only about 1/3 of the fat pad. Our result was expected, as there had been only one cycle of expression of the ECR3E protein. We believe that this is not sufficient enough to produce a significant effect on the tissue.

At 9 weeks the ductal system occupies about 2/3 of the fat pad of the mammary gland. At this time, we noticed a difference in the number of alveoli formed in the transgenic animal compared to wild type animals. The number of alveoli was decreased compared to the wild type littermate. This result corresponds well with the effect of active TGF-β on the mammary epithelia previously published from other groups (Pierce et al., 1993). Therefore, this result is encouraging with respect to potential effects of the transgene on tumor formation.

Analysis of the mammary glands in 6 month old virgin mice did not show significant differences in the appearance of the ducts and alveoli. At this stage, the ductal system occupies the whole fat pad, and is well spread and branched. It is possible that at this point the differences are not noticeable because the gland has been fully developed for some time and can compensate for the effects seen at 9 weeks. We are now working to examine the development of the

mammary gland in the period before and after 9 weeks to determine the exact time when expression of the transgene affects alveoli formation. At this point, it appears that expression of the transgene does not affect the branching of the ductal epithelium.

Because expression of WAP is high during lactation, we tried to look at the organization of the mammary glands during lactation. This proved to be very difficult to do by whole mount analysis since the glands and alveoli are filled with milk and so swollen that, after staining, it is not possible to determine with certainty any differences. To circumvent this problem, we are trying to do analysis of involuted glands. This is achieved by letting the females nurse their litters for a week, after which the pups are removed and the females maintained for different times, sacrificed and the glands analyzed. We expect that this will provide a better picture, as the majority of the milk and milk proteins will not be present and the ducts and alveoli will be easily visible. Our initial experiments indicate that there is a more rapid involution of the distal alveoli in the transgenic mice. This may indicate a more pronounced apoptosis because of heightened TGF-β levels.

In the next year, we will also initiate breeding of the WAP-ECR3E-RHA MMTV-neu #202 mice. These animals will be characterized for tumor induction vs. control MMTV-neu #202 mice as well as animals fed tamoxifen as described in the original application.

TGF-β and Breast Cancer Induction **Brukner Dabovic, Branka**

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Key Research Accomplishments

- Analysis of ductal branching and alveoli formation in origin WAP-ECR3E-2HA mice.
- Establishment of WAP-ECR3E-2HA x MMTV-neu mouse colony.

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